9-Hydroxycrisamicin A, a New Cytotoxic Isochromanquinone Antibiotic Produced by *Micromonospora* sp. SA246

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(Received for publication February 24, 1997)

9-Hydroxycrisamicin A, a new cytotoxic isochromanquinone antibiotic, was isolated from a soil microorganism SA246 which was identified as *Micromonospora* sp. The molecular formula of 9-hydroxycrisamicin A was determined as $C_{32}H_{22}O_{13}$ based on the HRFAB-MS analysis, and the structure was determined by various NMR experiments. 9-Hydroxycrisamicin A showed weak antimicrobial activity against Gram-positive bacteria and strong cytotoxic activity against some human cancer cell lines such as SK-OV-3 (ovarian), HCT15 (colon), SK-MEL-2 (melanoma), A549 (lung), XF498 (central nervous system) with ED_{50} of 0.47~0.65 μg/ml.

A soil microorganism, SA246 classified as *Micromonospora* sp., was found to produce a complex of antibiotic components. The major component of this complex was identified as crisamicin A[1~3], an isochromanquinone antibiotic, and as another major one, 9-hydroxycrisamicin A (Fig. 1) was purified by silica gel chromatography and HPLC at weakly acidified condition. It showed similar physico-chemical properties with crisamicin A, and shared the same molecular weight, *m/z* 615, of crisamicin C[4], an epoxide derivative of crisamicin A. In this paper, we describe the taxonomy of the producing organism, fermentation, isolation, structure elucidation and biological properties of 9-hydroxycrisamicin A.

Materials and Methods

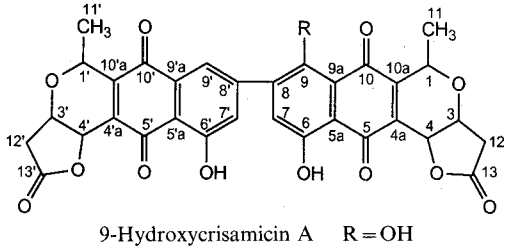
Taxonomy

The 9-hydroxycrisamicin A producing strain SA246 was isolated from a soil sample collected at a greenhouse in Taejon city, Korea. Taxonomic studies of the strain SA246 were carried out principally according to the methods of WILLIAMS *et al.*[5] and International *Streptomyces* Project (ISP)[6]. The carbon utilization pattern of SA246 was determined by the method of SHIRLING and GOTTLIEB[6]. Analysis of whole-cell hydrolyzates was performed according to the methods of SCHAAL[7]. Spore formation and spore surface ornamentation were observed with a scanning electron microscope (Model S-430, Hitachi), and classified as described by DIETZ and MATHEW category[8].

Fermentation

A loopful of a slant culture of SA246 was inoculated into 100 ml of seed culture medium (0.5% soluble starch, 0.05% aspartic acid, 0.05% $K_2HPO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$, pH 7.3) in a 500 ml Erlenmeyer

Fig. 1. Structures of 9-hydroxycrisamicin A and crisamicin A.



9-Hydroxycrisamicin A R=OH
Crisamicin A R=H

flask. After 5 days of incubation at 27°C on a rotary shaker (150 rpm), 1 ml of this seed culture was transferred to 100 ml of the same medium in a 500 ml flask and incubated at 27°C for 14 days on a rotary shaker (160 rpm). The cultivation of strain SA246 was carried out until the culture became dark brown.

Physico-chemical Properties

UV-visible spectrum was measured with a Shimazu UV-260 spectrophotometer. IR spectrum was obtained in KBr using a Analect fx-6160 FT-IR spectrophotometer. FAB and HRFAB-MS spectra were measured on a JEOL HX 100 mass spectrometer. The various NMR spectra in $CDCl_3$ were obtained on a Bruker NMR spectrometer at 400 MHz. Melting point was measured with a Fisher Johns melting point apparatus. TLC analysis was done on Merck Silica gel 60 F_{254} plates (250 μm).

Antimicrobial Activity

Antimicrobial activity was determined by agar dilution method using Mueller-Hinton and Sabouraud media for bacteria and fungi respectively. 9-Hydroxycrisamicin A was dissolved in DMSO and diluted serially in agar to give a final DMSO concentration of 0.5% in each test plate (55 × 12 mm). Final 9-hydroxycrisamicin A concentrations ranged from 100 to 0.39 μg/ml.

Cytotoxicity on Tumor Cells

Cytotoxicity of 9-hydroxycrisamicin A against human cancer cell lines was tested by SRB method[9]. Each cell line was diluted serially with RPMI media containing 5% FCS, and 0.2 ml of it was inoculated into well of tissue culture microplate. 9-Hydroxycrisamicin A was dissolved in DMSO and added to each well to give a final DMSO concentration of 0.1%. The results are expressed as ED_{50} values which are concentrations of 9-hydroxycrisamicin A that inhibit cell growth by 50%.

Results and Discussion

Taxonomy of the Producing Strain

The growth characteristics of the strain SA246 is presented in Table 1. Of all the media tested, yeast-malt extract agar supported the maximum growth. Colonies on yeast-malt agar medium showed dark brown diffusible pigment. The aerial mycelium is not formed on any medium tested.

Chemical analysis of whole cell hydrolysates of SA246 demonstrated the presence of *meso*-diaminopimelic acid as a component of cell wall. Gelatin, skim milk, and starch were readily hydrolyzed by broth cultures and D-mannitol, L-rhamnose, and sucrose were utilized as carbon source (Table 2). Electron microscopic examination (Fig. 2) of strain SA246 revealed branched vegetative mycelium bearing monospores (0.7 ~ 1.1 μm in diameter) with smooth spore surface.

The taxonomic characteristics of the strain SA246 were compared with those of type strains listed in "BERGEY's Manual of Systematic Bacteriology", volume IV[10]. And from the cultural characteristics of strain SA246 including lack of aerial mycelium, production of brown diffusible pigment, and production of single spores on short sporophores, and cell wall constituent, we tentatively identified this strain as *Micromonospora* sp. and named as *Micromonospora* sp. SA246. The more detailed taxonomic experiments are under study.

Isolation

As shown in Fig. 3, the whole culture broth was separated into mycelial cake and culture filtrate by centrifugation. The mycelial cake was extracted with 70% aqueous acetone, and the extract was filtered and concentrated *in vacuo* to remove organic solvents. This was combined with the culture filtrate and extracted successively with ethyl acetate without pH control. The dark red color extract was concentrated under reduced pressure. The dried material was dissolved in a small

Table 1. Cultural characteristics of strain SA246.

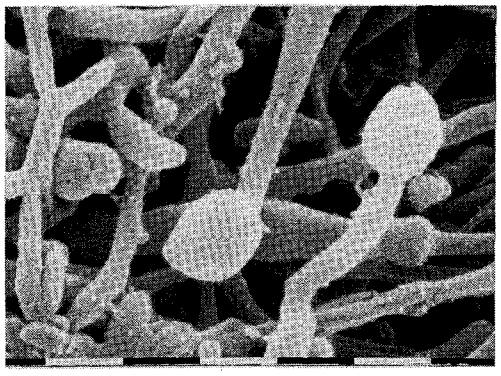
Medium	Growth	Reverse color	Aerial mycelium	Soluble pigment
Yeast-malt extract	Good	Pale brown	None	Pale brown
Oatmeal	Moderate	Pale brown	None	None
Inorganic salts starch	Poor	Pale yellow	None	None
Glycerol-asparagine	Poor	Pale yellow	None	None
Peptone-yeast-iron	Poor	Pale yellow	None	None
Tyrosine	Poor	Pale yellow	None	None
BENNET's	Poor	Pale yellow	None	None

Table 2. Morphological and physiological characteristics of SA246.

Spore	Single spores on short sporophores Smooth surface, 0.7~1.1 μm
Cell wall constituent	*meso*-DAP
Gelatin liquefaction	Positive
Skim milk hydrolysis	Positive
Starch hydrolysis	Positive
Carbohydrate utilization	
L-Arabinose	Negative
D-Fructose	Negative
D-Galactose	Negative
myo-Inositol	Negative
D-Mannitol	Positive
Raffinose	Negative
L-Rhamnose	Positive
Sucrose	Positive
Cellulose	Negative
D-Xylose	Negative
Cellobiose	Negative
Melibiose	Negative

Fig. 2. Scanning electron micrograph of strain SA246.

Bar represents 1.0 μm.



amount of chloroform-methanol (90:2, v/v) and chromatographed on a silica gel column with chloroform-methanol (90:2, v/v) and chloroform-methanol-acetic acid (90:3:0.1, v/v). The active material was efficiently eluted by latter solvent system and concentrated under reduced pressure to form red powder. The crude active red powder was further purified by preparative silica gel TLC with chloroform-methanol-acetic acid (90:2:0.1, v/v) followed by reversed phase HPLC (C18) using acetonitrile-water-acetic acid (65:35:0.1) as mobile phase.

Physico-chemical Properties of 9-Hydroxycrisamicin A

The physico-chemical properties of 9-hydroxycrisamicin A are summarized in Table 3. 9-Hydroxycrisamicin

Fig. 3. Isolation procedure for 9-hydroxycrisamicin A.

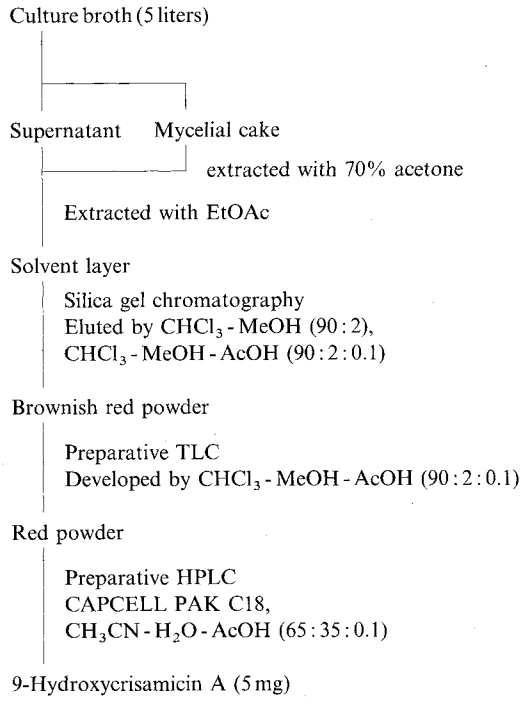


Table 3. Physico-chemical properties of 9-hydroxycrisamicin A.

Appearance	Red powder
MP (°C)	260 (dec)
Molecular formula	$C_{32}H_{22}O_{13}$
FAB-MS (*m/z*)	615 $(M+H)^+$
HRFAB-MS (*m/z*)	
Found	615.1115
Calcd.	615.1139
$[\alpha]_D^{20}$	+43° (*c* 0.14, $CHCl_3$)
UV $\lambda_{max}^{CH_3CN}$ nm (ε)	436 (5,538), 263 (17,906), 227 (31,858)
IR ν_{max}^{KBr} cm^{-1}	3000, 1789, 1648, 1621
Rf value on TLC*	0.52
Solubility	
Soluble (in acidic condition)	DMSO, CH_3CN, EtOAc, $CHCl_3$

* Silica gel TLC (Merck Art. 5715): $CHCl_3$-MeOH-AcOH (90:2:0.1).

A exhibits acid-base indicator properties. It thus showed red color in acidic solutions, and blue color at alkaline condition. During silica gel column chromatography, blue colored 9-hydroxycrisamicin A containing materials did not move in the $CHCl_3$-MeOH solvent system, but when other solvent system containing AcOH was used as mobile phase, the red colored 9-hydroxycrisamicin A abundant fractions were efficiently eluted. 9-Hydroxycrisamicin A was obtained as a red powder which decomposed at 260°C. It is soluble in DMSO, CH_3CN,

EtOAc, $CHCl_3$ at weakly acidic condition, but insoluble in most other organic solvent and water in neutral and alkaline pH. The UV-visible spectrum showed maxima at 436 (ε 5,538), 263 (ε 17,906), and 227 (ε 31,858) nm in weakly acidic CH_3CN. The pH-dependant spectral shift could not be measured due to its hard solubility. The IR spectrum showed the presence of three typical carbonyl groups, γ-lactone (1789), quinone (1648) and hydrogen bonded quinone (1621 cm^{-1}). Based on HRFAB-MS spectrometry the molecular weight of 9-hydroxycrisamicin A was determined to be 614.1816 and a molecular formula of $C_{32}H_{22}O_{13}$ was assigned.

Structure Assignment

The above physico-chemical properties and ^1H NMR spectrum of 9-hydroxycrisamicin A were very similar to those of crisamicin A, which was also produced by the present strain SA246. The molecular formula of crisamicin A was $C_{32}H_{22}O_{12}$, and 9-hydroxycrisamicin A was determined to be $C_{32}H_{22}O_{13}$ by the high resolution-FAB mass measurement. This result indicated that 9-hydroxycrisamicin A was a crisamicin A derivative with an additional oxygen. ^1H NMR spectrum of 9-hydroxycrisamicin A showed three hydrogen-bonded hydroxyl groups at 13.08, 12.50 and 11.86 ppm, three aromatic methine (two of them were *meta*-coupled each other), six oxygenated methine, two methylene and two methyl signals. The DEPT and ^{13}C NMR signals of 9-hydroxycrisamicin A included four quinone carbonyl carbons (187.6, 182.5, 178.4 and 178.3 ppm), two carbonyl carbons (174.5 and 174.4 ppm), three aromatic methine carbons (132.7, 126.1 and 121.1 ppm), three oxygenated sp^2 quaternary carbons (165.4, 164.5 and 162.2 ppm), ten sp^2 quaternary carbons and ten sp^3 carbons. The direct comparison of ^1H NMR spectrum and molecular formula of 9-hydroxycrisamicin A with those of crisamicin A suggested that 9-hydroxycrisamicin A replaced a *meta*-coupled aromatic methine proton of crisamicin A with a hydrogen-bonded hydroxyl group. This was supported by the presence of three oxygenated sp^2 quaternary carbons in the ^{13}C NMR spectrum. The HMQC data assigned all proton-bearing carbons and ^1H-^1H COSY experiment revealed five partial structures (Fig. 4). The structure of 9-hydroxycrisamicin A was established as shown in Fig. 4 by the HMBC data. Two ester groups (174.4 and 174.5 ppm) long-range correlated with the protons at 4.75 and 3.01, and 4.73 and 3.00 ppm were connected to C-4 and C-4′, respectively, to be γ-lactone moieties as suggested from IR spectrum. Also the three- or four-bond long range couplings from the protons at 5.10, 5.28, 7.57 and 7.89 ppm to two quinone carbonyl carbons at 182.5 and 187.6 ppm, and from the protons at 5.20, 5.33 and 7.39 ppm to quinone carbonyl carbons at 178.4 and 178.3 ppm were observed, indicating

Fig. 4. ^1H-^1H COSY and HMBC correlations for 9-hydroxycrisamicin A.

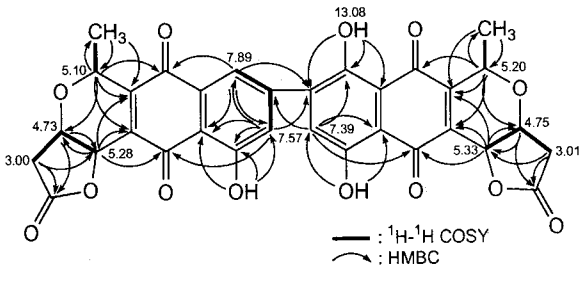


Table 4. ^1H and ^{13}C NMR spectral data for 9-hydroxycrisamicin A in $CDCl_3$.

Positions	^{13}C chemical shifts	^1H chemical shifts
1	67.4	5.20 (q, 6.9)*
3	67.0	4.75 (dd, 5.1, 3.0)
4	69.0	5.33 (d, 3.0)
4a	134.2	
5	178.3	
5a	112.1	
6	165.4	
6-OH		12.50 (s)
7	132.7	7.39 (s)
8	142.0	
9	164.5	
9-OH		13.08 (s)
9a	112.9	
10	178.4	
10a	149.8	
11	19.1	1.60 (d, 6.9)
12	37.5	3.01 (dd, 17.7, 5.1)
		2.74 (d, 17.7)
13	174.4	
1′	67.4	5.10 (q, 6.9)
3′	67.2	4.73 (dd, 5.1, 3.0)
4′	69.2	5.28 (d, 3.0)
4a′	135.2	
5′	187.6	
5a′	115.2	
6′	162.2	
6′-OH		11.86 (s)
7′	126.1	7.57 (d, 1.6)
8′	143.1	
9′	121.1	7.89 (d, 1.6)
9a′	132.2	
10′	182.5	
10a′	151.6	
11′	19.2	1.56 (d, 6.9)
12′	37.6	3.00 (dd, 17.7, 5.1)
		2.73 (d, 17.7)
13′	174.5	

* Proton resonance multiplicity and coupling constant (J = Hz) in parentheses.

Table 5. Antimicrobial activity of 9-hydroxycrisamicin A.

Organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	25
Bacillus subtilis IAM 1069	12.5
Sarcina lutea	6.25
Mycobacterium phlei IFO 3158	25
Corynebacterium lilium	25
Streptomyces scabies	25
Escherichia coli AB 1157	>100
Pseudomonas aeruginosa IFO 13130	>100
Candida albicans IAM 4905	>100
Saccharomyces cerevisiae IFO 1008	>100
Mucor ramannianus IAM 6218	>100
Aspergillus niger ATCC 9642	>100
Penicillium chrysogenum ATCC 12690	>100

the presence of two isochromanquinone moieties in structure of 9-hydroxycrisamicin A. A hydroxyl proton at 13.08 ppm showed the long-range correlations to sp^2 quaternary carbons at 164.5 and 142.0 ppm that were long-range coupled with the aromatic protons at 7.39, and 7.89 and 7.57, respectively, and thus the structure of 9-hydroxycrisamicin A was completely assigned as 9-hydroxy substituted crisamicin A, as shown in Fig. 1. The ^1H and ^{13}C NMR spectral data are summarized in Table 4.

Antimicrobial and Cytotoxic Activity of 9-Hydroxycrisamicin A

The antimicrobial spectrum of 9-hydroxycrisamicin A was tested against Gram-positive and Gram-negative bacteria, and fungi by agar dilution method. 9-Hydroxycrisamicin A showed a weak antimicrobial activity againt Gram-positive bacteria with minimal ihhibitory concentration ranging from 6.25 to 25 μg/ml and no activity aginst Gram-negative bacteria, yeast and fungi (Table 5).

The antitumor activity of 9-hydroxycrisamicin A against human tumor cell lines is shown in Table 6. 9-Hydroxycrisamicin A exhibited strong growth inhibitory effect on some tumor cell lines such as A549, SK-OV-3, SA-MEL-2, XF498, and HCT15.

Acknowledgment

This study was supported in part by funds from the Korea Science and Engineering Foundation, grant No. 941-0600-008-2.

Table 6. Cytotoxicity of 9-hydroxycrisamicin A on tumor cells.

Cells	ED_{50} (μg/ml)
A549 (lung)	0.54
SK-OV-3 (ovarian)	0.47
SK-MEL-2 (melanoma)	0.53
XF498 (central nervous system)	0.65
HCT15 (colon)	0.52

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